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13. ABSTRACT (Maximum 200 Words)

In year 2 of this grant, we have made more progress toward meeting our stated objectives. For Objective 1, we have; A. completed the studies establishing the dose and time course for progesterone in mediating neuroprotection against kainate-induced seizure and cell death. The studies showed that ovariectomized rats replaced with progesterone at physiological ranges reduced neuronal damage from seizures in proportion to the steroid's ability to reduce seizure activity. This activity is optimal at low to moderate progesterone dose. B. physiological levels of estrogen did not influence kainic acid seizures, but markedly reduced the neuronal damage that normally accompanies the seizures. C. have begun to study the interactions between estrogen and progesterone in influencing seizures and brain damage from persistent seizure activity, as well as investigating the mechanisms by which the steroids produce their effects. For Objectives 2 and 3, we have; obtained evidence that addition of progesterone (at concentrations of 5 and 10 μM) protects rat cerebellar neurons against glutamate-induced excitotoxic neuronal death. Addition of estradiol (1 μM) also protected rat cerebellar neurons against glutamate-induced excitotoxic neuronal death. Identified a novel posttranscriptional mechanism of regulation of mitochondrial gene expression that may play a role in the regulation under pathological conditions. We are extending these studies to examine the effects of hormones on the regulation of mitochondrial gene expression and the effects of estrogen plus progesterone on glutamate-induced excitotoxicity.

14. SUBJECT TERMS
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(4) INTRODUCTION:

Progesterone and Neuroprotection

Excitotoxicity contributes significantly to neuronal death and neurological impairment after both acute and chronic injuries; e.g., ischemia, head trauma, neurotoxicity, and stress related disorders. It is becoming increasingly clear that reproductive hormonal status can modulate brain excitability; generally, estrogen increases and progesterone decreases excitability to a number of stimuli, both natural and artificial. As women become more active in the military, issues of the interaction of cyclic changes in gonadal steroids on injury incurred during military service become relevant. Recent evidence indicates that the gonadal steroid progesterone greatly attenuates the excitotoxicity that leads to neuronal cell loss. The mechanisms whereby progesterone exerts such protective actions are not defined. The working hypothesis for the proposed studies is that progesterone treatment can protect or intervene against military-related brain injury due to toxicity, head trauma, and ischemia and that one mechanism of neuroprotection involves upregulation of mitochondrial gene expression.

(5) BODY:

The **specific hypotheses** to be tested are as follows:

1. **Progesterone protects against neuronal death caused by kainic acid-induced status epilepticus *in vivo* and excitotoxicity-induced neuronal death *in vitro*.**

2. **Excitotoxic levels of glutamate down-regulate the expression of mitochondrial genes, e.g., cytochrome oxidase, both *in vitro* and *in vivo*.**

3. **A mechanism of neuroprotection by progesterone is the up-regulation of mitochondrial gene expression, thereby increasing the resistance of neurons to excitotoxicity.**

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APPROVED STATEMENT OF WORK

Year 1

    Objective 1

- Complete studies establishing dose-time course for progesterone.

- Complete studies examining estrogen co-administration on progesterone's neuroprotectiveness.

- Begin establishing dose and time course for Finasteride and/or RU486 administration.

    Objective 2 and 3

- Continue measurements of COX III RNA, COX protein and COX enzyme activity on histological samples generated by experiments described in Objective 1.

- Complete establishment of dose-response relationships for glutamate and chloramphenicol (including 2 exposure regimens), COX expression and cell death using primary neuronal cultures.

- Begin measurements of cellular mitochondrial respiration, Ca^{2+} uptake and cytochrome c release.

Objective 1: Completion of studies establishing dose-time course for progesterone seizure/neuroprotection, and the initiation of studies determining the effect of estrogen on kainic acid-induced seizures and brain damage.

Is the reduction in seizures by progesterone correlated with the reductions in cell death in the hippocampus?

    Having established the optimal dose of kainic acid (KA) and survival time post-seizure for reliable induction of seizures and for producing clear damage to neurons, the analysis of

seizure activity in ovariectomized rats that received blank capsules or capsules containing progesterone was completed. Studies of animals that were ovariectomized and received estrogen were added. These studies established that progesterone at plasma concentrations of between 20 and 40 ng/ml significantly reduced seizure activity.

Study of effects of estrogen alone were deemed necessary as a prerequisite for assessing whether the combined application of estrogen and progesterone produced different effects than those observed with progesterone alone. As before, all experiments were conducted using ovariectomized (OVX) female rats (Sprague Dawley, Zivic Miller). At the time of ovariectomy animals were implanted with blank pellets or pellets (Innovative Research Laboratories, Sarasota FL) that were designed to deliver 15 or 40 pg/ml for a period up to 12 days. Seven days after implantation, animals were injected with KA at a dose of 8.5mg/kg i.p; controls received saline. Behavioral observations were conducted from 0-6 hr post-injection and seizure behaviors were scored using the Lothman and Collins Seizure Activity Scale (LCSAS) as described in our previous progress report. The results of the behavioral analysis determined that at doses of progesterone that yielded plasma levels of 20-40 ng/ml , seizures were significantly reduced (Figure 1A). At higher concentrations (above 40 ng/ml), progesterone did not reduce seizure scores. Estrogen's effects were quite different. Unlike progesterone, estrogen at either high (40 pg/ml, mimicking proestrus) or low (15 pg/ml, diestrus 1) doses failed to alter seizure severity (Figure 1A).

The animals were then prepared for anatomical analysis. Animals were sacrificed 96 hr post KA by an overdose of pentobarbital (100 mg/kg, i.p.) followed by perfusion with 2.5% acrolein in 4% paraformaldehyde. The brains were removed, sunk in 20% sucrose, cut on a freezing microtome at 35μm, and stained immunocytochemically using an antibody against a

neuron specific marker (Neuron nuclear antigen, NeuN). An observer blind to the conditions of the animals analyzed the anatomical data. The analysis of tissue to determine the extent of damage to the entorhinal cortex and hippocampal CA fields and the impact of progesterone or estrogen on the damage was performed by scoring the number of regions in which obvious cell loss was encountered with in the CA fields (CA1, CA2/3, hilus) and/or entorhinal cortex. Scores of 0 indicated no damage; scores of 1 to 4 reflected the number of different areas exhibiting damage. Statistical significance was determined by Kruskal Wallis non-parametric analysis. A 'p' value < 0.05 was considered significant. If the overall analysis showed significance post-hoc Mann Whitney tests were used to determine which groups differed. The quantification of damage is given in Figure 1B and the histology is shown in Figure 2.

An example of the hippocampal neuron patterns in a control animal that received saline in place of KA (no seizures) is shown in Figure 2A. Note the clear neuron layers of CA1, CA2/3 and the hilus. Ovariectomized rats with no hormone replacement treated with KA on average had seizure scores of 3 and this level of seizures was associated with clear neuron losses in all the CA fields (Figure 2B, stars). Animals that received low (Figure 2C) or high (Figure 2D) estrogen replacement prior to KA exposure, which had seizures similar in intensity to the ovariectomized only group of rats, had very normal neuronal patterns in their hippocampus that were indistinguishable from the animals that did not receive any kainate (Figures 1B and 2A). These data suggest that estrogen treatment dissociate the relationship between seizures and brain damage by preventing seizures from damaging neurons.

The results prompted the question of whether progesterone's ability to reduce brain damage was dependent upon its actions as an anticonvulsant or whether it too had protective effects beyond its ability to reduce seizures. Examination of the neuronal patterns of damage

within the entire population of progesterone-treated rats suggested that, neuron damage was tightly linked to the seizure severity. Figure 2D-F depicts progesterone-replaced rats that had no seizures (Figure 2D), a moderate seizure score (Figure 2E), and a score similar to those of the ovariectomized KA treated rats that did not receive hormonal replacement (Figure 2F). Note that the extent of damage increased with the severity of seizures in the progesterone treated rats. A correlation analysis was performed to determine if the extent of brain damage could be predicted by the severity of seizure behavior. The results are shown in Figure 3. Animals that did not receive any hormone replacement (Figure 3A) and animals that received progesterone treatment (Figure 3B) had a strong significant relationship between the severity of seizures and the extent of brain damage. This indicated that the ability of progesterone to protect the brain from seizure-induced damage depends on the steroid's ability to block seizures. In contrast, after estrogen replacement (Figure 3C), the presence of seizures did not correlate with brain damage, and irrespective of seizure severity, most animals has little or no brain damage. From these data, we suggest that progesterone exert its effects by reducing the source of the brain damage, specifically seizures. In contrast estrogen exert its effects very differently by protecting brain tissue from the cascade initiated by the seizures.   Together, these studies established that administration of progesterone can protect animals from seizures produced by kainate, and that a relatively lower dose of progesterone is effective in conveying this effect.   We are currently extending these studies to examine the potential neuroprotective effects of even lower doses of progesterone. In addition, we are beginning studies to examine the effects of co-administration of estrogen on the neuroprotective effects of progesterone.

Objective 2 and 3:

- **Continuation of the determination of COXII mRNA, protein and enzyme activity in the tissue generated in Objective 1.**

These studies are currently underway. We have obtained and screened 2 antibodies generated against the COX enzyme. Since comparisons among groups of animals for mRNA levels and quantitative analyses of protein levels require that all groups be screened in the same assay, the analysis of the effects of co-administered estrogen and progesterone will first be completed before completing the COX analyses.

- **Complete establishment of dose-response relationships for glutamate, COX expression and cell death using primary neuronal cultures.**

Experiments were done to test the protective effect of progesterone and estrogen against glutamate-induced neuronal death in cellular models of excitotoxicity. We used rat cerebellar granule neurons as a model of excitotoxicity. Levels of mitochondrial DNA-encoded subunit mRNA of oxidative phosphorylation system (OXPHOS) were also measured in rat cerebellar neuronal cultures during excitotoxicity to test the hypothesis that *excitotoxic levels of glutamate down-regulate the expression of mitochondrial genes, e.g., cytochrome oxidase.*

MATERIALS AND METHODS

Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies (NIH guide for the Care and Use of Laboratory Animals, NIH publication no. 85-23,1985).

Primary rat cerebellar cultures:

Cerebellar granule cell cultures were prepared from 7-day old Sprague-Dawley rats (Schousboe A et al. 1989). Neurons were seeded at a density of 2×10^5 cells/cm^2 in 6-well tissue culture

chambers coated with poly-L-lysine (MW 30 000 - 700 000) and cultured in Eagle's Basal Medium supplemented with Earle's salts, 10% inactivated fetal calf serum, 25 mM KCl and gentamycin (50 ng/ml). To prevent growth of glial cells, cytosine arabinoside (10 μM) was added to the cultures 24h after seeding.

Excitotoxicity:

Cultures were exposed to glutamate (50 - 250 μM) in a Locke solution (134-mM NaCl, 25 mM KCl, 4 mM NaHCO$_3$, 5 mM HEPES, 2.3 mM CaCl$_2$ and 5 mM glucose) for 30 min in the presence of 10 μM glycine (Ankarcrona et al. 1995). After exposure to glutamate, the cells were washed and kept in the old culture medium without glutamate for up to 24 hr. Control cultures were treated with the vehicle for the same time period as that of glutamate treated cells.

Drugs:

In experiments with cell cultures, progesterone and estrogen (17 β-estradiol) were dissolved in 60% alcohol and the final alcohol concentration was less than 0.01%. Cultures were treated with the vehicle alone were used as controls. Progesterone was used at a final concentration from 1 to 10 μM. Estrogen was used at a final concentration from 0. 1 to 10 μM.

Cell viability:

Cell viability was determined using a two-color fluorescence assay based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability – intracellular esterase activity and plasma membrane integrity. Viable granule cell neurons were quantified after staining of cells with cell-permeant calcein AM (2 μM). Nonviable neurons were quantified with cell-impermeant propidium iodide (10 μg/ml). The number of PI positive (nonviable) cells to the total number of cells in a field was determined.

RNA analysis

Rat cerebellar granule neurons were exposed to glutamate (100 µM) for 30 min in the presence or absence of progesterone (10 µM) as described. After exposure to glutamate, the cells were washed and kept in the old culture medium without glutamate for up to 24 hr. At various periods of time up to 24h, cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium and total RNA was isolated using the Qiagen RNeasy kit. Total RNA was subjected to northern blot analysis.

Two µg of total RNA was separated on 1.2% formaldehyde-agarose gel and transferred to GeneScreen Plus (NEN Life Sciences) using controlled vacuum (The Hybaid vacu-aid). Prehybridization and hybridization were done with Hybridizol reagent (Hybridizol I and II mixed 4 to 1 ratios, Oncor, MD, USA). The blots was prehybridized at 42°C for 16 h, then hybridized for 48 h at 42°C with $[^{32}P]$-labeled probe (Chandrasekaran et al. 1994). The blots were washed with increasing stringency and the final wash was performed at 65°C with 0.2 X SSC (1 X SSC = 150 mM sodium chloride and 15 mM sodium citrate) and 1% SDS (sodium dodecylsulfate). The blots were exposed to X-ray film (Bio-max MS, Kodak, NY, USA) with an intensifying screen for 45 min to 2 days at -70°C. The level of hybridized RNA was quantified using an image analysis program (NIH image 1.57 program, Wayne Rasband). The probe for evaluation of mtDNA transcripts was created by PCR amplification of mtDNA using specific primers (nt 8861-14549 of mtDNA). This probe hybridizes to COX III, ND3, ND4L, ND4, ND5 and ND6 mRNAs (Murdock et al. 1999). To control for equal loading and transfer of RNA, the blots was reprobed with β-actin or 18S rRNA.

Statistical analysis

Results are expressed as the mean ± S.E.M. Differences between controls and test samples were evaluated by ANOVA: Fischer's F-test was first used to compare between-groups and within-groups variance; if the former was significantly ($p<0.05$) higher than the latter, individual groups were compared by Tukey's test for multiple comparisons.

RESULTS

Effect of glutamate on cell death in cerebellar granule neurons:

We showed in the previous year report that the neurotoxicity produced by a 30min exposure to glutamate in Locke solution increased with increasing concentrations of glutamate (60 to ± 7% with 100 µM glutamate and 90 ± 5% with 250 µM glutamate; measured on sister cultures in 5 different preparations). The glutamate-induced neurotoxicity was blocked by the selective NMDA-receptor antagonist MK-801 (10 µM) and by the glycine antagonist 7-chlorokynurenic acid (10 µM). The results established that transient (30 min) exposure to glutamate (100 and 250 µM) produced reliable and consistent neuronal death after 24h.

Progesterone and estrogen protect against glutamate-induced excitotoxic cell death in rat cerebellar granule neuronal cultures:

Using the protocol of 30min exposure to glutamate, we evaluated the protective effect of addition of progesterone against neuronal death. In the previous report we showed that addition of progesterone up to 1 µM failed to provide significant protection against neuronal death. In this study, we tested the effect of progesterone at higher concentrations and also the effect of estrogen on glutamate-induced excitotoxicity. Cerebellar granule neurons were exposed to

glutamate (10 to 500 μM) for 30 min in the absence or presence of progesterone (1, 5 and 10 μM). Significant protection against glutamate-induced excitotoxic neuronal death was observed with progesterone at 5 and 10 μM concentrations with no protection at lower concentrations, 1 μM (Figures 4 and 5). The bright-field and PI fluorescence showing the morphology of the neuronal cultures is shown in Figure 4. The quantification of the protection is shown in Figure 5. The results obtained with estrogen on glutamate-induced excitotoxicity are shown in Figures 6 and 7. Addition of 17 β-estradiol at a concentration from 1 μM significantly protected cerebellar granule neurons against glutamate-induced neuronal death (Figure 7). The bright-field and PI fluorescence showing the morphology of the neuronal cultures is shown in Figure 6. Thus, our results suggest that both progesterone and estrogen protect rat cerebellar granule neurons against excitotoxic neuronal death, but at a concentration much higher than the physiological level.

Glutamate decreases mitochondrial gene expression and that addition of progesterone protects against it.

We have initiated experiments to test the hypothesis that *excitotoxic levels of glutamate down-regulate the expression of mitochondrial genes, e.g., cytochrome oxidase.* Rat cerebellar granule neurons were exposed to glutamate (100 μM) for 30 min. Total RNA was isolated at various periods of time up to 24h. The total RNA was subjected to northern blot analysis with mtDNA specific probe as described in 'Materials and Methods'. The results are shown in Figure 8. Addition of glutamate decreased rapidly the expression of mtDNA-encoded COX and NADH dehydrogenase subunit genes. Reprobing of the blots with β-actin showed no specific decreases suggesting that the decrease is not due to loss of RNA or due to differences in RNA loading (not

shown). Addition of progesterone protected against glutamate-induced decreases in mitochondrial gene expression (Figure 8).

To gain insight in to the mechanism of mitochondrial gene expression, we investigated transcriptional and posttranscriptional regulation of mitochondrial gene expression in response to increasing and decreasing energy demand in rat pheochromocytoma PC12 cells. These results are published in Molecular and Cellular Biochemistry (copy enclosed). Treatment of PC12 cells either with ouabain, an inhibitor of Na/K-ATPase, or with monensin, a sodium ionophore, decreased the steady-state levels of mtDNA-encoded COX III mRNA by 50%, 3-4h after addition of the drugs. No significant reduction in mtDNA-encoded 12S rRNA or nuclear DNA-encoded β-actin mRNA were observed. Removal of the drugs restored the normal levels of COX III mRNA. Determination of half-lives of COX III mRNA, 12S rRNA and β-actin mRNA revealed a selective decrease in the half-life of COX III mRNA from 3.3h in control cells to 1.6h in ouabain-treated cells, and to 1h in monensin-treated cells. *These results suggested the existence of a mechanism of posttranscriptional regulation of mitochondrial gene expression that is independent of the energetic status of the cell and that may operate under pathological conditions.* We are extending these studies to investigate whether similar mechanism is responsible for glutamate-induced decreases in mitochondrial gene expression. We are also testing the effect of progesterone and estrogen on mitochondrial gene expression. The rationale for this is that estradiol has been shown to increase levels of several mtDNA-encoded mRNA in hepG2 cells (Chen et al. 1998) and stimulate expression of adenine nucleotide translocator ANT1 messenger RNA in female rat hearts (Too et al. 1999).

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These studies showed that addition of progesterone and estrogen at micromolar concentrations protect rat cerebellar neurons against glutamate-induced excitotoxic neuronal death. We are currently extending these studies to cortical neuronal cultures and also examining the effects of co-administration of estrogen and progesterone on glutamate-induced excitotoxicity. The mechanism of glutamate-induced effects on mitochondrial gene expression and the direct effects of progesterone and estrogen on mitochondrial function including its mitochondrial gene expression are being studied.

FIGURE LEGENDS

Figure 1. Effects of progesterone and estradiol on the reduction of severity of kainate-induced seizures and extent of damage. A) Seizure scores and B) extent of damage were determined in saline (S) or kainic acid (K) treated ovariectomized rats bearing blank capsules (B) or progesterone (P) capsules or estradiol (E) pellets. Progesterone treated animals with plasma levels of <20 ng/ml, 20-40 ng/ml, 40-60 ng/ml or >60 ng/ml were grouped together. Estradiol treated animals that produced plasma levels of 15 or 40 pg/ml were grouped together. Values are expressed as the mean score or extent of damage (number of regions exhibiting neuronal loss) ± S.E.M. *Significantly different from B+K, $p<0.05$.

Figure 2. Reduction of hippocampal neuronal death by progesterone and estradiol following kainate treatment. Photomicrographs show Nissl stained hippocampus sections in ovariectomized (ovx) rats. (A) ovx+saline; (B) ovx+kainate with high seizure score; (C) ovx +low estradiol (E)+ kainate with the same seizure score as B; (D) ovx+high E+kainate also with seizure score comparable to B; (E) progesterone (P)-treated rat low seizure score; (F) P-treated rat with moderate seizure score and (G) P-treated rat with high seizure score similar to B-D. Note that there is little neuronal damage in the E-treated rats compared to that seen in animals with no hormones (stars), and that the damage in P-treated rats was proportional to the severity of seizures.

Figure 3. Correlation between the extent of damage and seizure score. Correlation analysis between the extent of damage and seizure score showed significant relationship in no hormone (A) and in progesterone-treated rats (B) and an absence in estradiol-treated rats (C).

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Figure 4. Effect of addition of progesterone on glutamate-induced neuronal death. At 8 DIV, the neuronal culture was replaced with Locke solution. Cultures were treated with the vehicle or with progesterone (10 μM) for 30 min. Glutamate (100 μM) and glycine (10 μM) were then added to the culture medium for 30 min. The neurons were then cultured in normal medium without glutamate for the next 24h. Photographs were made under bright field and propidium iodide fluorescence microscopy under dark field. Representative fields from a single experiment is shown in the figure but the experiments were repeated at least three times with identical results. (A) Culture treated with vehicle, (B) culture treated with glutamate [glutamate (100 μM) plus glycine (10 μM)]; and (C) culture treated with glutamate in the presence of progesterone. Note that the addition of progesterone protected rat cerebellar cultures against glutamate-induced excitotoxic neuronal death.

Figure 5. Dose-response protection by progesterone against glutamate-induced excitotoxicity. At 8 DIV, the neuronal culture was replaced with Locke solution. Progesterone was added at concentration of 1 or 5 or 10 μM for 30 min prior to the addition of glutamate and glycine (10 μM). Viable neurons are stain green with calcein AM whereas nonviable neurons stain positive with propidium iodide. At each tested concentration, the mean and SEM of rat cerebellar neurons that stained positive with propidium iodide are expressed as a percentage of the total cell count. Sister cultures were used for each concentration and the experiments were repeated in 3 different culture preparations. Mean ± SEM are shown. ***$p<0.001$; **$p<0.01$; *$p<0.05$, Tukey's test for multiple comparisons.

Figure 6. Effect of addition of 17 β-estradiol on glutamate-induced neuronal death. At 8 DIV, the neuronal culture was replaced with Locke solution. Cultures were treated with the vehicle or with 17 β-estradiol (10 μM) for 30 min. Glutamate (100 μM) and glycine (10 μM) were then added to the culture medium for 30 min. The neurons were then cultured in normal medium without glutamate for the next 24h. Photographs were made under bright field and propidium iodide fluorescence microscopy under dark field. Representative fields from a single experiment is shown in the figure but the experiments were repeated at least three times with identical results. (A) Culture treated with vehicle, (B) culture treated with glutamate [glutamate (100 μM) plus glycine (10 μM)]; and (C) culture treated with glutamate in the presence of 17 β-estradiol. Note that the addition of estradiol protected rat cerebellar cultures against glutamate-induced excitotoxic neuronal death.

Figure 7. Dose-response protection by 17 β-estradiol against glutamate-induced excitotoxicity. At 8 DIV, the neuronal culture was replaced with Locke solution. Estradiol was added at concentration of 0.1 or 1 or 10 μM for 30 min prior to the addition of glutamate and glycine (10 μM). Viable neurons are stain green with calcein AM whereas nonviable neurons stain positive with propidium iodide. At each tested concentration, the mean and SEM of rat cerebellar neurons that stained positive with propidium iodide are expressed as a percentage of the total cell count. Sister cultures were used for each concentration and the experiments were repeated in 3 different culture preparations. Mean ± SEM are shown. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$, Tukey's test for multiple comparisons.

Figure 8. Progesterone Protects Against Glutamate-induced Decrease in mtDNA-encoded mRNAs. Neuronal cells were exposed to glutamate (100 μM) and glycine (10 μM) in the presence or absence of progesterone (10 μM). Total RNA was isolated at the indicated periods of time and subjected to Northern blot analysis. The hybridization signal was quantified by image analysis of autoradiograms. The relative changes in mRNA was related to zero time samples. Each point is the average of two separate experiments.
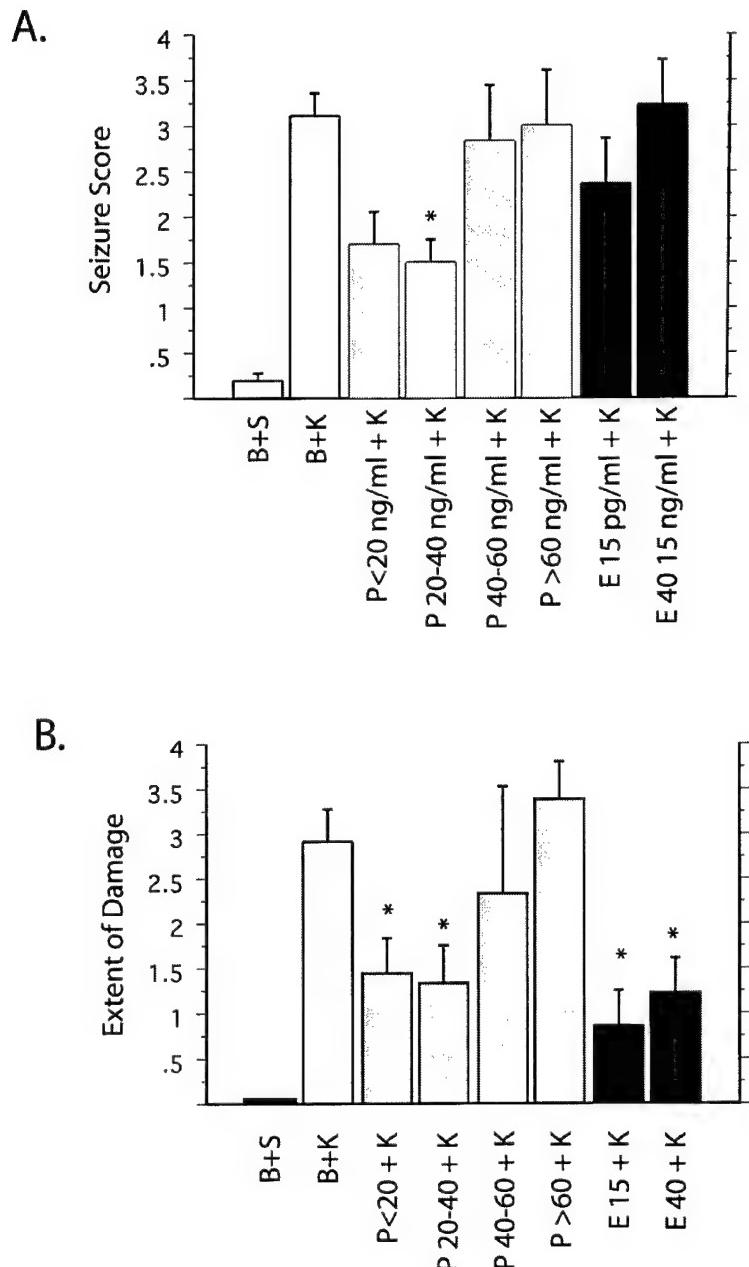
Figure 1. Effects of progesterone and estradiol on the reduction of severity of kainate-induced seizures and extent of damage.

Figure 2. Reduction of hippocampal neuronal death by progesterone and estradiol following kainate treatment.

A.

No Hormone

$R^2 = .667$
$P < 0.05$

Spread / Seizure Score

B.

Progesterone

$R^2 = .639$
$P < 0.05$

Spread / Seizure Score

C.

Estrogen

$R^2 = -0.11$
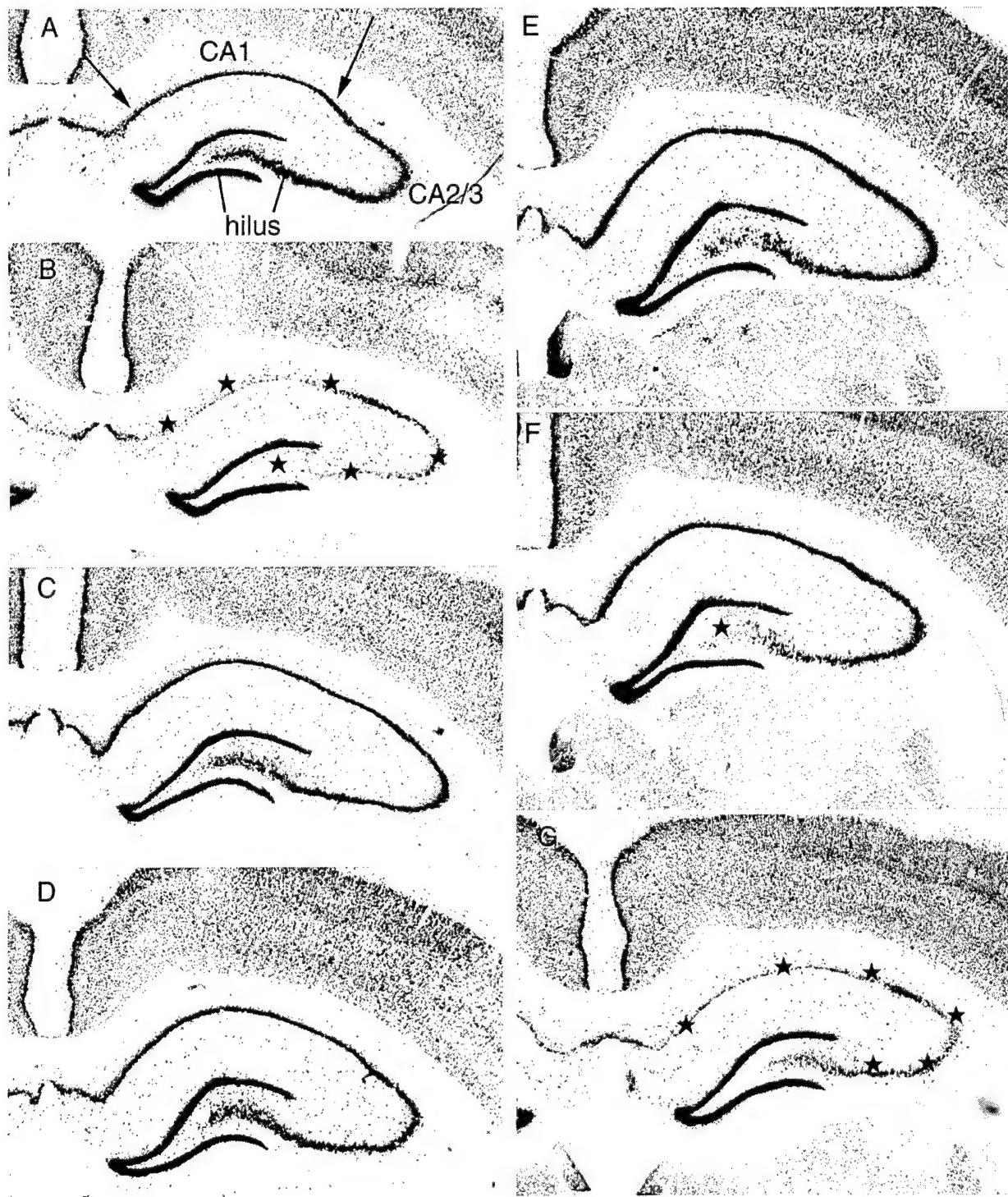
Spread / Seizure Score
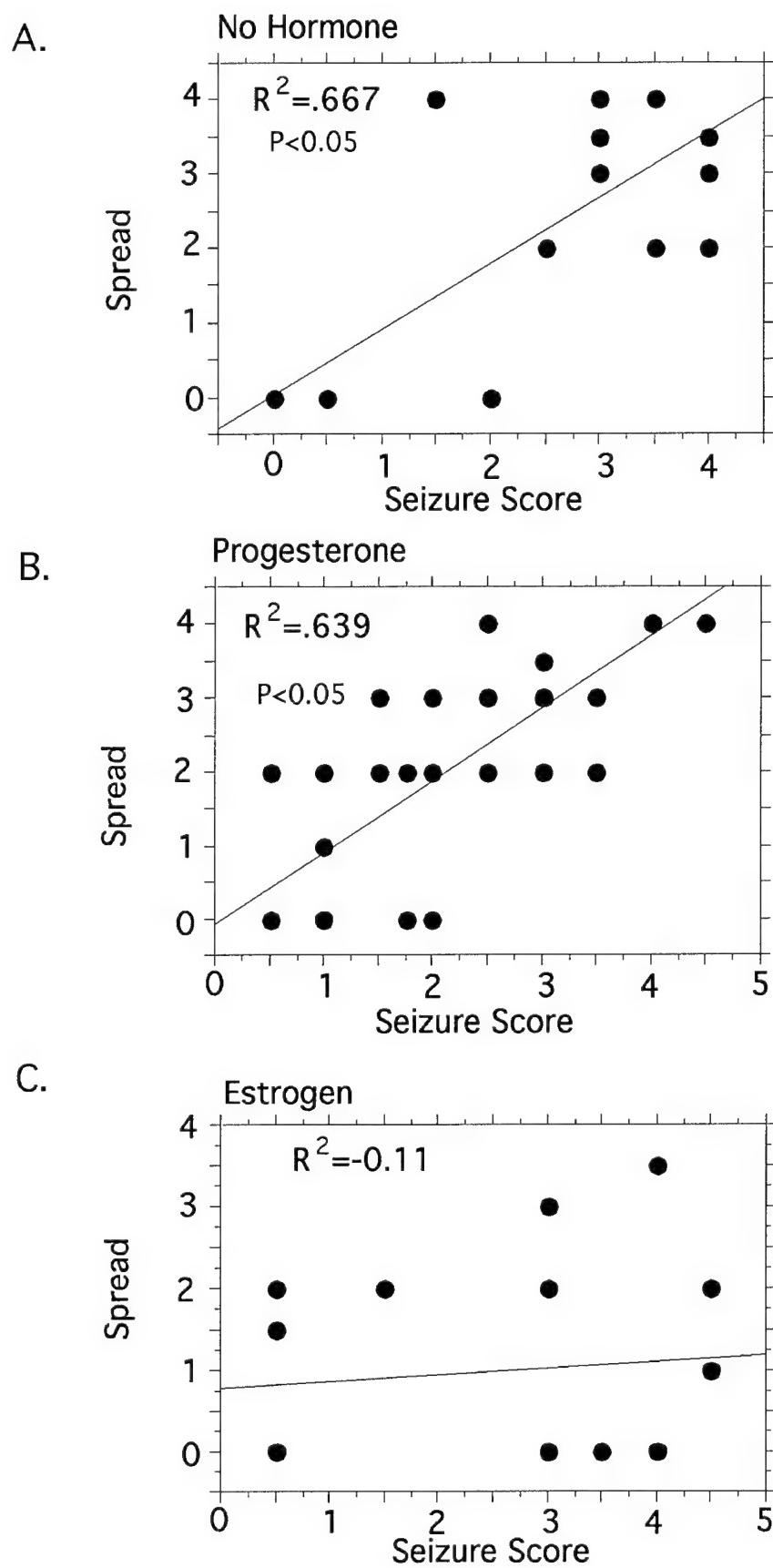
Figure 3. Correlation between the extent of damage and seizure score.
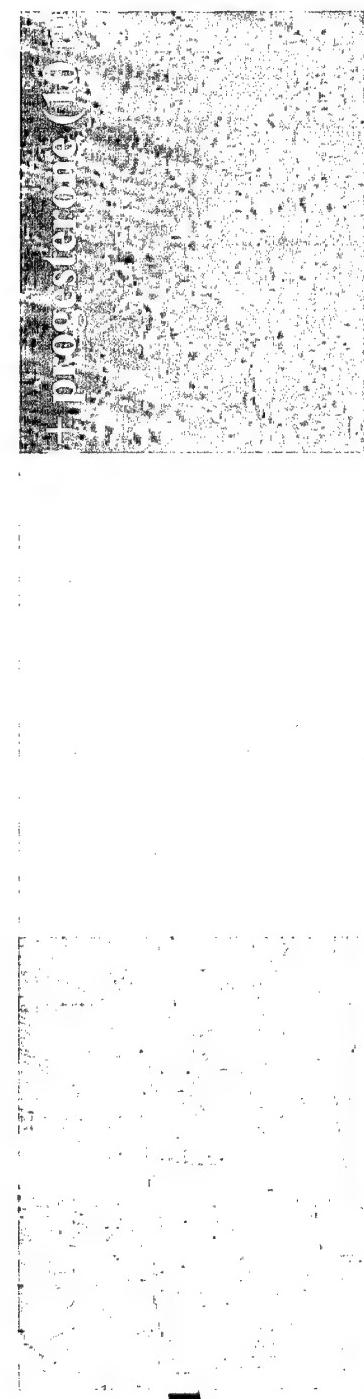
Bright-field

Propidium-Iodide

Progesterone (10)

A   B   C
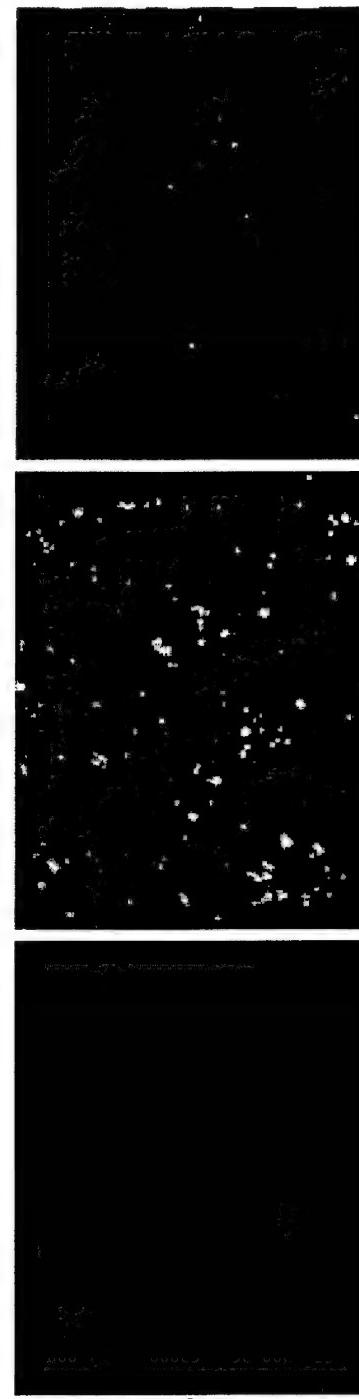
Figure 4. Progesterone protects against glutamate-induced excitotoxicity in rat cerebellar granule neurons

Figure 5. Dose-response protection by progesterone against glutamate-induced excitotoxicity

Bright field

Propodium iodide

A   B   C
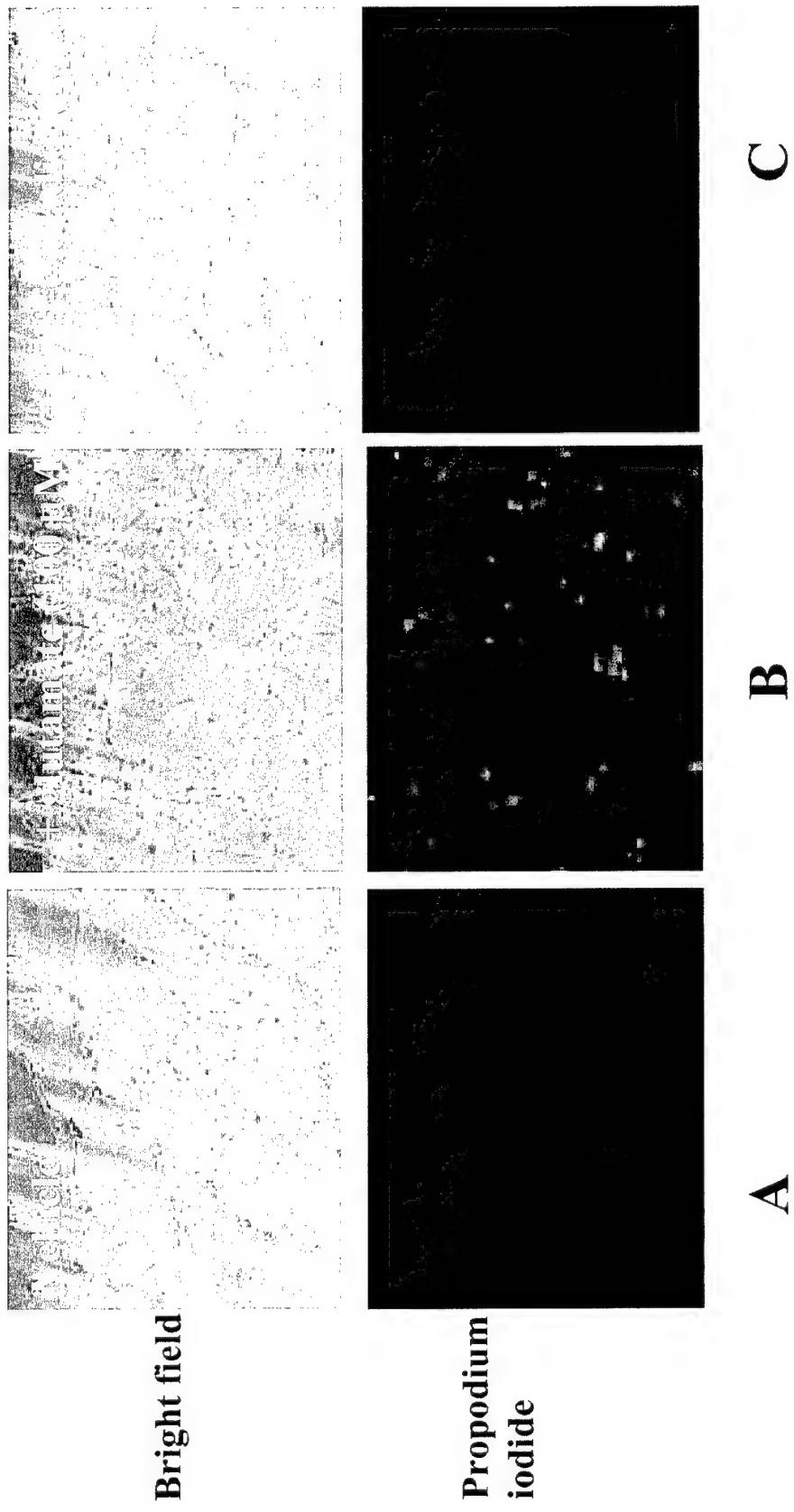
Figure 6. 17 β-estradiol protects against glutamate-induced excitotoxicity in rat cerebellar granule neurons
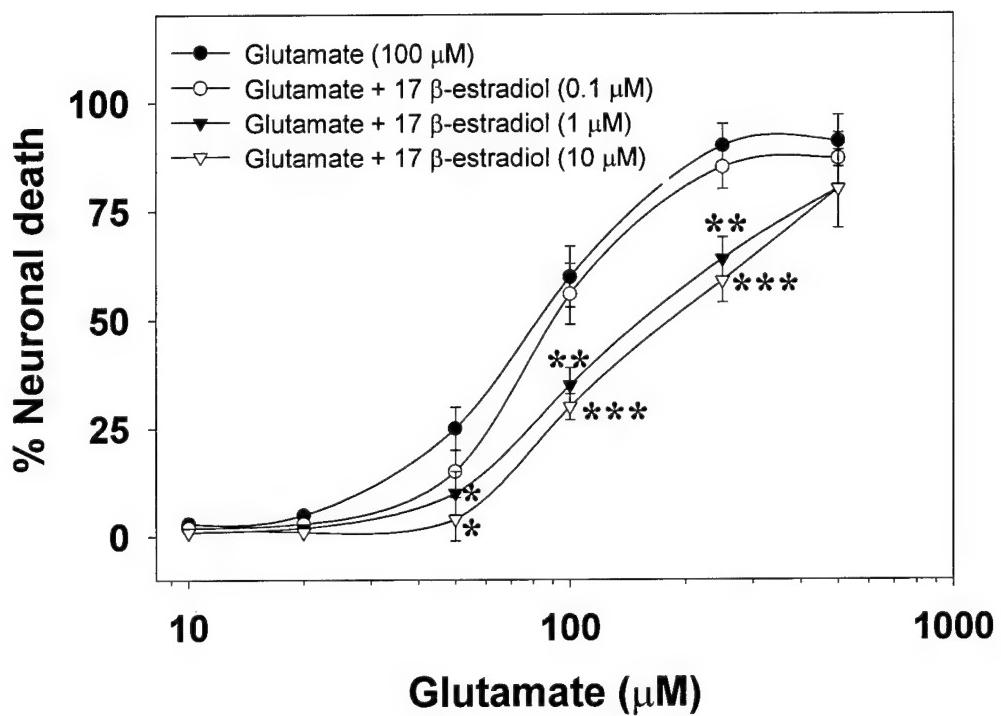
Figure 7. Dose-response protection by 17 β-estradiol against glutamate-induced excitotoxicity
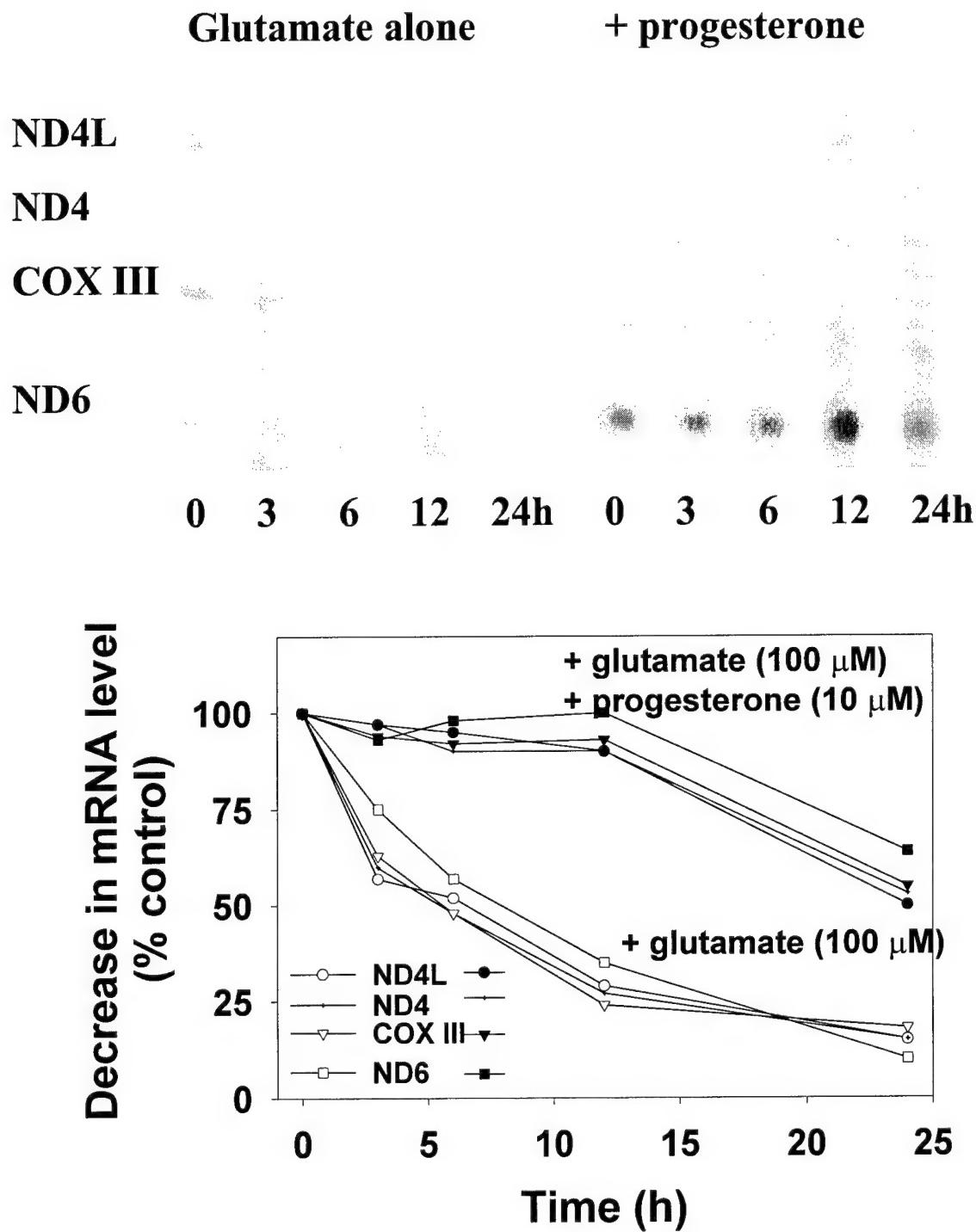
Figure 8. Progesterone protects against glutamate-induced decrease in mtDNA-encoded mRNAs

6) Key Research Accomplishments

- Established the relationship between the dose of progesterone in both altering seizures produced by kainic acid and producing neuron damage to the hippocampus.

- Established the relationship between the dose of estradiol (estrogen) in affecting seizures and brain damage produced by kainic acid.

- Evidences obtained to suggest that progesterone and estradiol (estrogen) protect against glutamate-induced excitotoxicity in rat cerebellar neuronal cultures.

- Identified a novel mechanism of posttranscriptional regulation of mitochondrial gene expression

- Evidences obtained to suggest a relationship between down-regulation of mitochondrial gene expression and glutamate-induced excitotoxicity.

(7) Reportable Outcomes

- **Manuscript**

Chronic exposure of neural cells to elevated intracellular sodium decreases mitochondrial mRNA expression. Chandrasekaran K, Liu LI, Hatanpaa K, Shetty U, Mehrabyan Z, Murray PD, Fiskum G and Rapoport SI. *Mitochondrion* 1(2), 141-150 (2001).

- **Abstracts**

Seizure severity and hippocampal damage following kainate are dissociated after estrogen but not progesterone treatment in rats. Hoffman GE, Moore N, Le WW, Fiskum G, Chandrasekaran K and Murphy AZ. *Soc. Neurosci, in press* (2001).

Pre-lethal inhibition of mitochondrial gene expression by elevating neural cell sodium with monensin, ouabain, veratridine or excitotoxic levels of glutamate and kainate. Chandrasekaran K, Mehrabyan Z and Fiskum G. *Soc. Neurosci, in press* (2001).

Inhibition of mitochondrial gene expression by elevating neural cell sodium with monensin, ouabain, veratridine or excitotoxic levels of glutamate and kainate. Chandrasekaran K, Mehrabyan Z and Fiskum G. *The fifth European Meeting on Mitochondrial Pathology, in press* (2001).

(8) Conclusions

Objective 1:

Established that ovariectomized rats replaced with progesterone at physiological ranges reduced neuronal damage from seizures in proportion to the steroid's ability to reduce seizure activity. This activity is optimal at low to moderate progesterone doses.

Established that ovariectomized rats replaced with physiological levels of estrogen did not influence kainic acid seizures, but markedly reduced the neuronal damage that normally accompanies the seizures.

We are now beginning to study the interactions between estrogen and progesterone in influencing seizures and brain damage from persistent seizure activity, as well as investigating the mechanisms by which the steroids produce their effects.

Objectives 2 and 3:

Established that transient (30 min) exposure to glutamate (100 μM) produced reliable and consistent neuronal death after 24 hr.

Evidences obtained that addition of progesterone (at concentrations of 5 and 10 μM) protect rat cerebellar neurons against glutamate-induced excitotoxic neuronal death.

Evidences also obtained that addition of estradiol (1 μM) protect rat cerebellar neurons against glutamate-induced excitotoxic neuronal death.

Identified a novel posttranscriptional mechanism of regulation of mitochondrial gene expression that may play a role in the regulation of mitochondrial gene expression under pathological conditions.

We are extending these studies to examine the co-administration of estrogen and progesterone on glutamate-induced excitotoxicity and also the effects of hormones on the regulation of mitochondrial gene expression.

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(10) Appendices:

1 Manuscript and 3 Abstracts

RAT FOREBRAIN DIGITONIN PREP

(synaptosomal + non-synaptosomal mitochondria)

Materials

Filled ice bucket

Homogenizer w/ pestles (A-loose pestle & B-Tight pestle)

Glass rod and pasteur pipettes

MS + EGTA Buffer

MS Buffer without EGTA

Thawed digitonin (10% stock in DMSO)

Preparative Centrifuge pre-cooled to 4º C (JA-20 Rotor)

MS + EGTA buffer

> 225 mM Mannitol
>
> 75 mM Sucrose
>
> 5 mM HEPES
>
> 250 µM EGTA
>
> 1 mg/ml Fatty acid free BSA
>
> pH 7.4

MS buffer without EGTA:

> Prepare MS buffer as above without EGTA

Hints: Make sure mitochondria (and tubes, homogenizer, and glass rod) are kept ice-cold at all times, make sure no ice comes in contact with mitochondria

Digitonin is used to permeabilize synaptosomes.

Procedure

1) Decapitate animal.

2) Cut open skull, remove forebrain, and cut off cerebellum (should be done as rapidly as possible ~30 sec).

3) Place forebrain in **MS + EGTA** buffer that has been chilling in a beaker on ice and mince brain rapidly with a good pair of scissors.

4) Rinse forebrain in **MS + EGTA** buffer to remove blood and add it to the homogenizer.

5) Homogenize 6 times with loose (A) pestle and 6 times with tight (B) pestle.

6) Divide the brain suspension into test tubes for centrifugation (3 test tubes). (Add buffer to balance tubes and use a fourth tube for balance).

7) **Centrifuge using Ja-20 rotor at 6,000 rpm (2800g) for 3min at 4° C.**

8) Place supernatant into chilled beaker.

9) Resuspend pellet in small volume of **MS +EGTA** buffer with glass pipette and add additional buffer to bring up the volume. Combine the pellets and add buffer as needed to balance the tubes.

10) **Centrifuge for 6000 RPM (2800g) for 3min at 4° C.**

11) Place supernatant into chilled beaker and discard the pellet.

12) The collected supernatant is now divided into 4 tubes and buffer is added as needed to balance.

13) **Centrifuge JA-20 for 14,000rpm (15,000g) for 8min at 4° C.**

14) Aspirate off supernatant and retain pellet. Squirt some buffer into each tube. Using a glass stirring rod and as gently as possible, brush pellet off the side of tube. Resuspend with glass pipette gently 2-3 times.

15) Combine resuspended pellets into one tube and then divide equally into 2 tubes, to distribute mitochondria evenly between the two tubes. Then add **MS+EGTA** buffer to about an inch from the top of the tubes.

16) Get tube of **10% Digitonin** (in DMSO) and add 40ul to each tube

17) Place parafilm over the tubes and invert 5 times.

18) **Centrifuge using JA 20 rotor at 14,000 (15,000g) for 10 min at 4° C.**

19) Aspirate the supernatant and the white cloudy layer around the brown pellet. Get the **MS buffer without EGTA**. Squirt a small amount of this buffer into each test tube and resuspend mitochondria gently with glass rod/pipette. Use transfer pipette to add MS buffer without EGTA to the resuspension to about an inch from the top of the tubes.

20) **Centrifuge using JA 20 rotor at 14,000 (15,000g) for 10 min at 4° C.**

21) Aspirate off supernatant. Place 2 drops of **MS buffer without EGTA** into one of the centrifuge tubes. Resuspend pellet carefully using glass rod and transfer to 2nd tube. Resuspend 2nd pellet and place into a chilled microfuge tube.

22) Protein concentration can be determined by using a Biuret assay with BSA standards.

Chronic exposure of neural cells to elevated intracellular sodium decreases mitochondrial mRNA expression

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Abstract

Regulation of expression of mitochondrial DNA- (mtDNA-) encoded genes of oxidative phosphorylation can occur rapidly in neural cells subjected to a variety of physiological and pathological conditions. However, the intracellular signal(s) involved in regulating these processes remain unknown. Using mtDNA-encoded cytochrome c oxidase subunit III (COX III), we show that its mRNA expression in a differentiated rat pheochromocytoma cell line PC12S is decreased by chronic exposure to agents that increase intracellular sodium. Treatment of differentiated PC12S cells either with ouabain, an inhibitor of Na/K-ATPase, or with monensin, a sodium ionophore, decreased the steady-state levels of COX III mRNA by 50%, 3–4 h after addition of the drugs. No significant reduction in mtDNA-encoded 12S rRNA and nuclear DNA-encoded β-actin mRNA were observed. Removal of the drugs restored the normal levels of COX III mRNA. Determination of half-lives of COX III mRNA, 12S rRNA, and β-actin mRNA revealed a selective decrease in the half-life of COX III mRNA from 3.3 h in control cells to 1.6 h in ouabain-treated cells, and to 1 h in monensin-treated cells. These results suggest the existence of a mechanism of posttranscriptional regulation of mitochondrial gene expression that is independent of the energetic status of the cell and may operate under pathological conditions. © 2001 Elsevier Science Ireland B.V. and Mitochondria Research Society. All rights reserved.

Keywords: Neural cells; Mitochondrial mRNA; Intracellular sodium

1. Introduction

Neurons depend on a high rate of mitochondrial oxidative metabolism to produce ATP (Erecinska and Silver, 1989). ATP is needed for ion pumping to restore the cellular membrane potential after depolarization. The mitochondrial respiratory chain

consists of five multisubunit oxidative phosphorylation (OXPHOS) enzyme complexes. Four of these OXPHOS complexes, including Complex IV (cytochrome oxidase (COX)), are bipartite in nature, consisting of subunits derived from both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). Mitochondrial DNA encodes 13 polypeptides, all of which are necessary for electron transport and OXPHOS. The large number of remaining subunits is specified by the nuclear genome. To form active enzyme complexes, both mtDNA- and nDNA-

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encoded subunits are required (Attardi and Schatz, 1988).

Neuronal activity and energy demand influence the expression of mitochondrial DNA-encoded genes. For example, under conditions of decreased neuronal activity induced by afferent impulse blockade, neuronal mitochondrial gene expression and COX enzyme activity are decreased (Wong-Riley, 1989; Wong-Riley et al., 1997). Removal of the afferent impulse blockade restores basal mitochondrial gene expression and COX activity (Wong-Riley, 1989; Wong-Riley et al., 1997). Such regulation occurs mainly at the transcriptional level (Wong-Riley et al., 1997; Zhang and Wong-Riley, 2000a,b). Using the in organello method, it was shown that high intramitochondrial ATP levels suppress transcription of mtDNA, explaining how energy demand can regulate mtDNA transcription (Gaines and Attardi, 1984; Enriquez et al., 1996a,b). Apart from transcriptional control, the primary regulation of mitochondrial gene expression is based on differences in RNA stability. Thus, although mitochondrial gene expression is a major component in the regulation of energy metabolism of the cell, the contribution of transcriptional and posttranscriptional mechanisms to the overall regulation of mitochondrial gene expression is not known (Kagawa and Ohta, 1990).

The goal of our present study was to probe the mechanism of regulation of mitochondrial gene expression under conditions of chronic exposure to two drugs that increase intracellular sodium ($[Na]$), but by two different independent mechanisms. Ouabain reduces cellular Na^+ efflux by inhibiting the Na/K-ATPase and would be expected to reduce cellular energy demand. Monensin is a Na^+ ionophore, which in contrast to ouabain, would increase the cellular energy demand through futile cycling of Na^+ across the plasma membrane. These expected effects on energy metabolism were confirmed by measurements of cellular ATP/ADP ratios and compared to the levels of mtDNA-encoded cytochrome oxidase subunit (COX III) mRNA in cultures of nerve growth factor (NGF-) induced differentiated PC12 neural cells/neuronal cultures. Both the levels and half-lives of COX III mRNA, mtDNA-encoded 12S rRNA, and nDNA-encoded β-actin mRNA were quantified. Our results indicate a selective decrease in mtDNA-encoded mRNA stability in both ouabain-

and monensin-treated cells, suggesting an Na^+-mediated mechanism of posttranscriptional regulation that is independent of the energetic status of the cell in neuronal cultures. A part of this work has been published as abstract (Liu et al., 1999).

2. Methods and materials

2.1. Cell culture

A morphological variant of rat pheochromocytoma PC12 cells (PC12S) that has the ability to grow in tissue culture dishes without polylysine treatment was used in experiments (Fukuyama et al., 1993). PC12S cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine, 7.5% heat inactivated fetal calf serum, 7.5% heat inactivated horse serum, and penicillin–streptomycin. Differentiation was induced by the addition of NGF (Life Technologies, MD, USA) at 50 μg/ml to the cell culture medium. We showed previously that the morphology of PC12S cells resembles that of sympathetic neurons after addition of NGF for 5 days (Fukuyama et al., 1993). Differentiated PC12S maintained in NGF for 10 days was used throughout the experiments.

2.2. Chemicals

All reagents and chemicals used were of the highest grade available from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of actinomycin D and ouabain were prepared in water, whereas monensin was dissolved in 95% ethanol. When ethanol was used as a solvent, appropriate control experiments were conducted using the vehicle alone. Ethanol concentrations were always <0.1%.

2.3. Experimental procedure

Cells grown in 60×15 mm dishes were treated either with ouabain, at a final concentration of 1 mM, or with monensin, at a final concentration of 100 nM. At timed points over a 6-h period, cells were washed with Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium and total RNA was isolated using the TRIzol reagent as recommended by the manufacturer (Life Technologies, MD,

USA). Total RNA was subjected to Northern blot analysis as described below.

The reversibility of the effect of ouabain and monensin on mitochondrial gene expression was evaluated by exposing the differentiated PC12S cells to the drugs for a period of 6 h. The cells were then washed three times with DPBS without calcium and magnesium, fresh DMEM growth medium with NGF was added, and total RNA was isolated at various times over a 24-h period and processed for Northern blot analysis.

The effect of ouabain or monensin on the stability of mtDNA- and nDNA-encoded transcripts was determined by adding the transcriptional inhibitor actinomycin D to the cultures at a final concentration of 5 μg/ml. After 1 h, either vehicle or ouabain or monensin was added. Total RNA was isolated at various times over an 8-h period and processed for Northern blot analysis.

2.4. RNA analysis

Ten μg of total RNA was run on a 1.2% formaldehyde agarose gel and transferred on to a GeneScreen Plus membrane as described by the manufacturer (Dupont, New England Nuclear, MA, USA). Prehybridization and hybridization were done with Hybridizol reagent (Hybridizol I and II mixed in the ratio of 4:1, Oncor, MD, USA). The blots were prehybridized at 42°C for 16 h, then [^{32}P]-labeled cytochrome oxidase subunit III (COX III) probe was added and hybridized for 48 h at 42°C (Chandrasekaran et al., 1994). The blots were washed with increasing stringency and the final wash was performed at 65°C with 0.2 × SSC (1 × SSC = 150 mM sodium chloride and 15 mM sodium citrate) and 1% sodium dodecyl sulfate (SDS). The blots were exposed to X-ray film (Biomax MS, Kodak, NY, USA) with an intensifying screen for 45 min to 2 days at −70°C. Probe was removed from the blots by placing them in boiling DEPC-treated water for 10 min. The blots were then rehybridized with a [^{32}P]-labeled control β-actin probe as described above. Finally, the blots were hybridized with 12S rRNA probe. The level of RNA hybridized was quantified using an image analysis program (NIH Image 1.57 program written by Wayne Rasband, NIH). To maintain measured intensities within the linear range, the blots hybridized with

different probes were exposed for different periods. The level of RNA was quantified from autoradiograms of lower exposure than was used for photography. Ratios of COX III mRNA to β-actin mRNA and 12S rRNA to β-actin mRNA were calculated (Chandrasekaran et al., 1994).

2.5. Probe preparation and labeling

Cytochrome oxidase subunit III, 12S rRNA and β-actin probes were prepared by isolating the cDNA insert from the plasmid clones (American Type Culture Collection, VA, USA). The cDNA fragments were gel purified and labeled with [^{32}P] dCTP using a random primed labeling kit (Pharmacia, NJ, USA). The labeled probes were purified using probe purification columns (Pharmacia, NJ, USA). Northern blots hybridized with either probe showed a single band of expected size, verifying the specificity of the probes.

2.6. Estimation of half-life of COX III mRNA, 12S rRNA, and β-actin mRNA

Ten μg of total RNA from cells treated with either vehicle or ouabain or monensin in the presence of actinomycin D was subjected to Northern blot analysis. The blots were hybridized with COX III, 12S rRNA and β-actin probes and the levels of the respective RNA species were quantified. Levels of β-actin mRNA are expressed as the percentage of β-actin mRNA remaining at each experimental time compared to zero time. Levels of COX III mRNA and 12S rRNA were calculated as the ratio of the respective species to the level of β-actin mRNA. At each experimental time, the RNA ratios are expressed as a percentage of the ratio at time zero. The half-lives were determined from the equation $t_{1/2} = 0.301/\text{slope}$ of the best-fit line ($\log_{10}$ remaining RNA versus time).

2.7. Measurement of ATP/ADP ratio

Differentiated PC12S cells were treated with vehicle or ouabain or monensin for various periods as described. Nucleotides were extracted using hot methanol (Shryock et al., 1986). Briefly, the cells were scraped in 5 ml of hot (75°C) 80% methanol containing 0.5 mM EDTA. The extract was centrifuged at 8000 × g for 10 min at 4°C. The supernatant was transferred to a fresh glass tube and evaporated to

dryness. The residue was dissolved in 1 ml distilled water, 0.5 ml chloroform was added, vortexed, and the samples were centrifuged at $2000 \times g$ for 4 min at 10°C. Fifty μl of the aqueous solution was injected on HPLC columns. The adsorbosphere nucleotide–nucleoside column (7 μm, 250×4.6 mm, Allteck) with two-solvent system was used for separation of ATP and ADP. Solvent A contained 60 mM $NH_4H_2PO_4$, 5 mM tetrabutyl-ammonium phosphate, pH 5.0 and solvent B contained methanol with 5 mM tetrabutyl-ammonium phosphate. The gradient of HPLC was from 90 to 64% of solvent A during a period of 20 min, then maintained for 10 min, then returned to 90% of solvent A for 10 min. The flow rate was 1 ml/min and the nucleotides were detected at 259 nM. The resolution of ADP and ATP were determined using external ATP and ADP standards. The peak areas were used to calculate the ratio of ATP to ADP.

2.8. Measurement of intracellular sodium

PC12 cells were differentiated with NGF (50 ng/ml) for 5 days. In order to measure the intracellular sodium concentration rather than the influx, the culture medium was replaced with a medium containing NGF and ^{22}Na (5 μCi or 185 KBq per ml) for another 5 days. Measurement of intracellular ^{22}Na showed that an equilibration between added radioactive label with the cold sodium in the medium was achieved within 24 h. The cells were then treated for various time periods with either the vehicle or monensin (final concentration: 100 nM). The reaction was terminated by aspiration of the medium and the cells were quickly washed twice with ice-cold DPBS and digested for 1 h in 0.5 ml of 1 M NaOH at room temperature. Cell digests were assayed for ^{22}Na contents by scintillation counter.

2.9. Statistical analysis and replication of results

The results presented are representative of at least three to five independent experiments. Where indicated, statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. The differences were considered significant when $P < 0.05$.

3. Results

3.1. Treatment of differentiated PC12S cells with ouabain or monensin alters the ratio of ATP/ADP

Ouabain, an inhibitor of the plasma membrane Na/K-ATPase, and monensin, an Na^+ ionophore, are useful tools for elevating $[Na]_i$ (Pressman and Fahim, 1982). This effect of monensin on differentiated PC12S cells was confirmed by incubating cell cultures in the presence of ^{22}Na. Exposure of cell cultures for 3 h to 100 nM monensin resulted in a 500% increase in ^{22}Na, from 30 ± 5 to 150 ± 12 pmol/μg protein. Exposure of cell cultures to ouabain (1 mM) for 3 h resulted in a 300% increase in ^{22}Na, from 30 ± 5 to 85 ± 9 pmol/μg protein (Pressman and Fahim, 1982).

The effect of ouabain and monensin on cellular ATP/ADP ratios was tested. Addition of ouabain caused a rapid and nearly 100% increase in the ATP/ADP ratio followed by a return to a ratio that was still significantly higher (50%) than that of vehicle-treated cells (Fig. 1). Addition of 100 nM monensin resulted in a sustained, approximately 40% decrease in ATP/ADP ratio. These results suggested that in differentiated PC12S cells, the Na/K-ATPase is one of the major consumers of ATP.
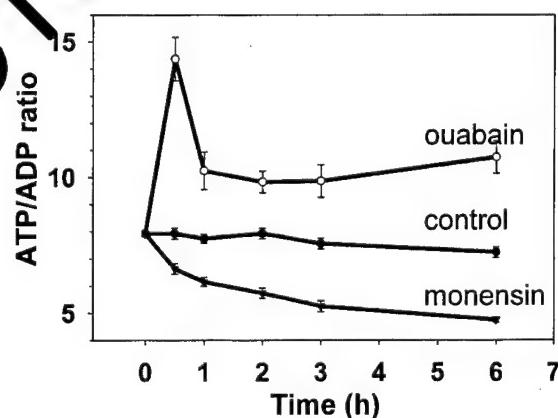

Fig. 1. Time course and extent of ouabain- and monensin-induced ATP/ADP ratio changes in differentiated PC12S cells. Extracts were prepared with hot methanol from cells that were treated with the drugs for various time periods. The nucleotide phosphates were resolved using HPLC and the ratio of ATP to ADP was determined from their peak areas. Each point is the mean± SEM of four separate experiments.

Inhibition of the sodium pump by ouabain reduces the consumption of ATP and thereby increases the ATP/ADP ratio. In contrast, influx of sodium ions by the ionophore, monensin, activates the pump, causing an increased consumption of ATP and a decreased ATP/ADP ratio. However, measurements of cell viability using trypan blue exclusion indicated no decrease in viability (>95% trypan blue exclusion) following at least 6 h exposure to either ouabain or monensin. Microscopic examination of the cells indicated that within 1 h after the addition of ouabain or monensin there was increased cell swelling. This was likely due to increased intracellular sodium ion $[(Na^+)_i]$, caused either by an inhibition of Na/K-ATPase (ouabain) or by an influx of sodium ions (monensin), accompanied by a passive influx of Cl^- and shifts in water content.

3.2. Chronic treatment of differentiated PC12S cells with ouabain decreases mtDNA-encoded COX III mRNA levels

To examine the effects of ouabain and monensin on mtDNA-encoded COX subunit III gene expression, it was first necessary to ascertain the steady-state level of COX III mRNA in PC12S cells treated with vehicle. PC12S cells were differentiated with NGF for 10 days. The cells were treated with the vehicle (water or ethyl alcohol (0.01%)) for various periods of time, total cellular RNA was isolated, and 10 μg aliquots were subjected to Northern analysis as described in Section 2. Blots of RNA were probed with mtDNA-derived cDNAs encoding COX III and 12S rRNA as well as nDNA-derived cDNA encoding β-actin. Levels of β-actin mRNA were determined to ensure that equivalent amounts of RNA were loaded and transferred into each lane in Northern blot analyses. The results showed that there was no evidence of any significant modulation of steady-state transcript levels of COX III, 12S rRNA, and β-actin genes in vehicle-treated PC12S cells (not shown).

Treatment of differentiated PC12S cells with the Na/K-ATPase inhibitor ouabain decreased the steady-state levels of mtDNA-encoded COX III mRNA (Fig. 2A,C). Mitochondrial DNA-encoded 12S rRNA, however, was unaffected by ouabain treatment (Fig. 2A,B). To ensure that the quantity of 12S-specific radiolabeled probes were not limiting in this experiment, serial dilutions of RNA from these cells

were subjected to dot-blot analysis with specific probes, and analysis confirmed that the steady-state quantity of 12S rRNA was indeed unaffected (data not shown). There was also no evidence of any significant ouabain-induced modulation of steady-state nDNA-encoded β-actin mRNA levels (Fig. 2A).
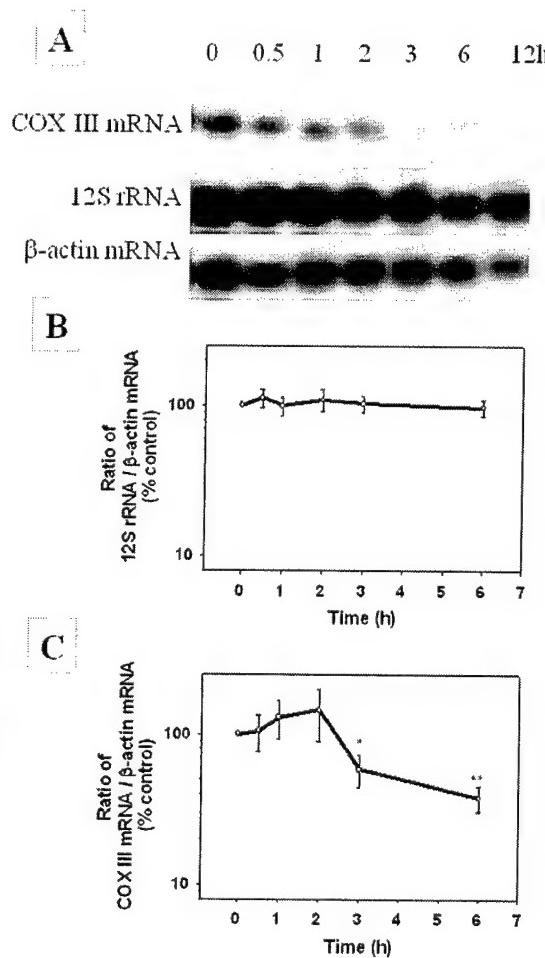

Fig. 2. The time course of changes in β-actin mRNA, 12S rRNA, and COX III mRNA levels in ouabain-treated cells. The β-actin mRNA, 12S rRNA, and COX III mRNA levels of differentiated PC12S cells exposed to ouabain (final concentration 1 mM) for the indicated periods were determined by Northern blot analysis and quantified by image analysis of autoradiograms. The relative changes in β-actin mRNA was related to zero time samples. The ratio of COX III mRNA to β-actin mRNA and 12S rRNA to β-actin mRNA ratio was calculated at each time point and was then related to the ratio of zero time samples. Each point is the mean ± SEM of three to five separate experiments. The asterisk indicates the significant difference from zero time samples ($P < 0.05$).

These results taken together with the elevation of the ATP/ADP ratio by ouabain shown in Fig. 1 suggested that inhibition of the sodium pump by ouabain decreased the consumption of ATP (energy demand) and consequently decreased the levels of mtDNA-encoded COX III mRNA.

3.3. Chronic treatment of differentiated PC12S cells with monensin decreases mtDNA-encoded COX III mRNA levels

If ouabain decreased mitochondrial gene expression by decreasing cellular energy demand, it follows that exposure of cells to a condition that increases energy demand should increase gene expression. Thus, cells were exposed to the Na^+ ionophore monensin, anticipating that the levels of COX III mRNA would increase due to the decrease in the ATP/ADP ratio (see Fig. 1) caused by cycling of Na^+ across the plasma membrane. Although there was a trend toward an increase in COX III mRNA following 1 h exposure to monensin, the net effect was a significant decrease 3 and 6 h after addition of monensin. Mitochondrial DNA-encoded 12S rRNA, however, was unaffected by monensin treatment (Fig. 3A,B). There was also no significant monensin-induced modulation of steady-state nDNA-encoded β-actin mRNA levels (Fig. 3A).

3.4. Removal of ouabain and monensin restores normal levels of mtDNA-encoded COX III mRNA

Differentiated PC12S cells were treated with ouabain or monensin for a period of 6 h. The cells were washed free of drugs and replaced with fresh medium. As shown in Fig. 4, removal of drugs restored the normal levels of COX III mRNA within 12 h, suggesting that the decrease in COX III mRNA was not due to the toxicity of ouabain and monensin. Moreover, cell viability as measured by trypan blue dye exclusion showed that the cells remained viable after addition of ouabain or monensin up to a period of 6 h. Estimation of mtDNA by dot-blot analysis showed no significant reduction in both ouabain- and monensin-treated cells compared to vehicle-treated cells, suggesting that the observed decrease in COX III mRNA was not due to loss of mitochondria (not shown).

3.5. Treatment of differentiated PC12S cells with ouabain or monensin decreases the stability of mtDNA-encoded COX III mRNA

To test whether the decrease in mtDNA-encoded
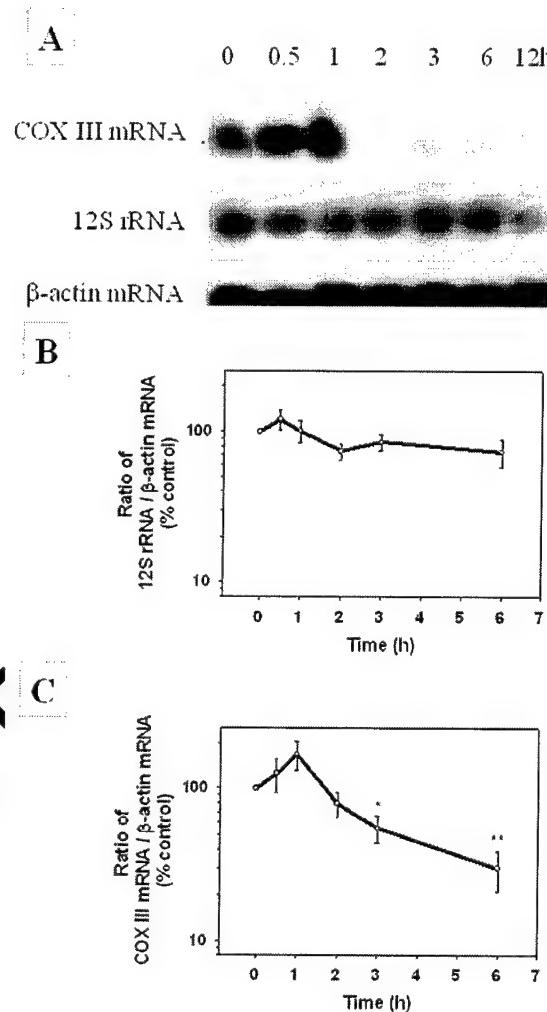
Fig. 3. The time course of changes in β-actin mRNA, 12S rRNA, and COX III mRNA levels in monensin-treated cells. The β-actin mRNA, 12S rRNA, and COX III mRNA levels of differentiated PC12S cells exposed to the vehicle for the indicated periods were determined by Northern blot analysis and quantified by image analysis of autoradiograms. The relative changes in β-actin mRNA were related to zero time samples. The ratios of COX III mRNA to β-actin mRNA and of 12S rRNA to β-actin mRNA were calculated at each time point and were then related to the ratio of zero time samples. Each point is the mean ± SEM of five separate experiments. The asterisks denote a significant difference of the sample from the zero time samples ($P < 0.05$).
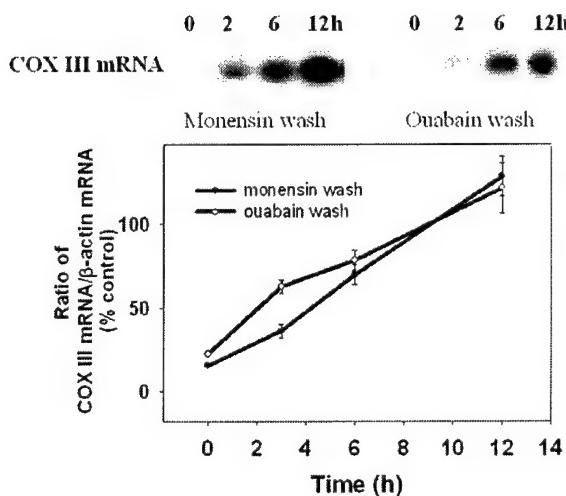
Fig. 4. Effect of the removal of ouabain and monensin on the ratio of COX III mRNA to β-actin mRNA. Differentiated PC12S cells exposed to ouabain and monensin for a period of 6 h. The cells were then washed free of drugs and were maintained in normal culture medium. The COX III mRNA and β-actin mRNA levels for the indicated periods were determined by Northern blot analysis and quantified by image analysis of autoradiograms. The relative change in COX III mRNA/and β-actin mRNA ratio was related to COX III mRNA/and β-actin mRNA ratio of zero time samples. Each point is the mean ± SEM of three separate experiments.

COX III mRNA in ouabain- or monensin-treated cells is due to increased degradation or decreased synthesis, we determined the half-life of COX III mRNA, 12S rRNA, and β-actin mRNA in control, ouabain-, and monensin-treated differentiated PC12S cells. Actino-mycin D has been previously shown to inhibit total cellular transcription in PC12 cells by more than 95%. To estimate half-lives, actinomycin D was added to differentiated PC12S cells and after 1 h vehicle, ouabain, or monensin was added. Total cellular RNA was isolated at various times over an 8-h period. Throughout this period, cell viability was not compromised and there was no substantial reduction in total RNA yield in the presence of actinomycin D. Fig. 5A shows autoradiograms of RNA isolated and probed with COX III and β-actin. Clearly, mRNA encoding COX III was significantly affected, decreasing the estimated $t_{1/2}$ from 3.3 h in control cells to 1.6 h in ouabain-treated cells and to 1 h in monensin-treated cells (Fig. 4C) respectively. No such decrease in half-lives were observed with mtDNA-encoded 12S rRNA (Fig. 4B) and nDNA-encoded β-actin mRNA (Fig.

4A) in both ouabain- and monensin-treated cells. Also, the estimated $t_{1/2}$ of COX III mRNA in control cells was much shorter (3.3 h) when compared to the $t_{1/2}$ of 12S rRNA (17 h) and β-actin mRNA (>17 h).
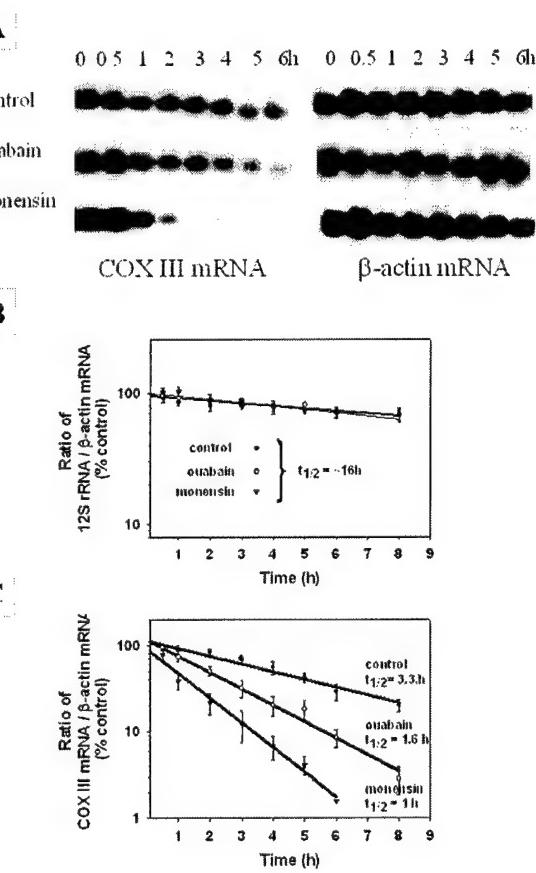

Fig. 5. Estimation of half-lives for β-actin mRNA, 12S rRNA, and COX III mRNA in control, ouabain-, and monensin-treated differentiated PC12S cells. Total cytosolic RNA was isolated from control, ouabain-, and monensin-treated cells at indicated time points after termination of transcription by actinomycin D, and 10 μg aliquots were subjected to Northern blot analysis as described in Section 2. Hybridization was quantified by image analysis of autoradiograms. Semi-log plots of transcript remaining versus time is depicted in the case of β-actin mRNA. In the cases of 12S rRNA and COX III mRNA, the ratios of 12S rRNA to β-actin mRNA and COX III mRNA to β-actin mRNA were calculated. The relative change in the ratio was related to the ratio of zero time samples. Each point is the mean ± SEM of three separate experiments.

4. Discussion

The results of the present study demonstrate that chronic treatment with both ouabain and monensin induced a selective decrease in mtDNA-encoded COX III mRNA expression in differentiated PC12S cells. No significant decreases were observed with mtDNA-encoded 12S rRNA and with nDNA-encoded β-actin mRNA in both ouabain- and monensin-treated cells. Moreover, estimation of mtDNA by dot-blot analysis showed no significant reduction in both ouabain- and monensin-treated cells compared to vehicle-treated cells. The observed decrease in COX III mRNA levels in both ouabain- and monensin-treated cells is, therefore, not due to either loss of mitochondria or a general breakdown of RNA. On the other hand, a similar reduction in both ouabain- and monensin-treated cells was observed with mtDNA-encoded COX subunit-I mRNA and -II mRNA (data not shown). Thus, the effect of ouabain and monensin appears to be specific for mtDNA-encoded mRNA and not for rRNA. This may relate to differences in the synthesis and stability between mtDNA-encoded mRNA and rRNA. A similar decrease in COX III mRNA with ouabain and monensin was observed also in undifferentiated and differentiated PC12 cells (T. Tom, personal communication), in a human neuroblastoma cell line SHSY5Y and in monensin-treated rat primary cerebellar granule neurons. Thus, it is unlikely that the choice of the cell culture could account for the ouabain- and monensin-induced response.

Addition of ouabain to differentiated PC12 cells led to a rapid and prolonged rise in the ATP/ADP ratio. In neural tissues, a major portion of the energy derived from metabolism is used to restore ionic gradients to resting levels and the Na/K-ATPase is one of the major consumers of ATP (Mata et al., 1980; Sokoloff, 1981; Erecinska and Silver, 1989). Consistent with these observations, our results show that an inhibition of Na/K-ATPase by ouabain decreases ATP consumption and increases the ATP/ADP ratio in differentiated PC12S cells.

We originally hypothesized that ouabain would reduce mitochondrial gene expression as a direct consequence of an elevated ATP/ADP ratio. In organello transcription experiments using isolated mitochondria show that mitochondrial RNA synthesis

can be regulated in response to changes in intramitochondrial ATP levels (Gaines et al., 1987; Enriquez et al., 1996b). High levels of intramitochondrial ATP suppress mtDNA transcription possibly by inhibiting mitochondrial RNA polymerase, presenting a mechanism by which energy demand could regulate mtDNA transcription (Enriquez et al., 1996b). Although the results demonstrating a reduction in COX III mRNA levels associated with increased ATP/ADP levels in ouabain-treated cells is consistent with these mechanisms of gene regulation, the observation that the estimated $t_{1/2}$ of COX III mRNA was decreased from 3.1 h in control cells to 1.6 h in ouabain-treated cells suggested that RNA degradation rather than synthesis was the primary site of control by ouabain. This result suggests that both transcriptional and posttranscriptional mechanisms are likely to be involved in the maintenance of steady level of mtDNA-encoded COX III mRNA.

The unexpected result of this study was the observed decrease in COX III mRNA in monensin-treated cells. Monensin is a Na$^+$-selective ionophore that causes Na$^+$ influx with a corresponding efflux of H$^+$ or K$^+$ in numerous cell types including PC12 cells (Pressman and Fahim, 1982; Shier and DuBourdieu, 1992). Under our experimental conditions, we observed a 500% increase in ^{22}Na after exposure of PC12 cell cultures for 3 h to 100 nM monensin (not shown). Ionic or pharmacological interventions that increase [Na$^+$]$_i$ levels cause significant induction of Na/K-ATPase activity and functional Na/K-pump sites (Wolitzky and Fambrough, 1986; Lingrel and Kuntzweiler, 1994). Addition of monensin to neuronal cells enhances cellular energy metabolism and increases ATP utilization by Na/K-ATPase, presumably to restore ionic gradients to resting levels (Mata et al., 1980). Accordingly, we found that the addition of monensin to differentiated PC12S cells decreased the ATP/ADP ratio, likely due to increased ATP consumption. We anticipated that with elevated ATP consumption, there would be an up-regulation of COX III mRNA. Though there was an increase in COX III mRNA levels in the first hour after addition of monensin, the levels of COX III mRNA subsequently showed a significant decrease. We interpret these findings to suggest that the initial increase represents a mechanism of increased expression due to increased energy demand. The decrease in COX III

mRNA following chronic exposure to monensin suggests the presence of an alternate mechanism for the regulation of mitochondrial gene expression that is independent of the energetic status of the cell and that overrides the normal regulation by energy demand. This decrease in mitochondrial gene expression under conditions of increasing energy demand is not unique to this system. For example, levels of mtDNA-encoded cytochrome oxidase subunit I (COX I) mRNA decreases within hours in CA1 neurons of gerbils after transient forebrain ischemia (Abe et al., 1993). This early decrease in COX I mRNA occurs in the absence of a decrease in mtDNA, suggesting impaired mitochondrial gene expression occurring at the level of transcription and/or turnover of mitochondrial mRNA (Abe et al., 1993). In this model system, the decrease in COX I mRNA occurs when the energy demand is high on these cells not only to restore ionic gradients to resting levels but also to maintain neuronal activity (Arai et al., 1986; Abe et al., 1993). A disproportionate decrease in mtDNA-encoded COX subunit mRNA in the absence of changes in mtDNA-encoded 12S rRNA is also observed in the brains of Alzheimer's disease (AD) patients (Chandrasekaran et al., 1994,1998; Hatanpääet al., 1996).

One explanation for the observed decrease in COX III mRNA but not of 12S rRNA is the relatively short half-life of COX III mRNA. Decay of COX III mRNA, 12S rRNA, and β-actin mRNA in the presence and absence of ouabain or monensin were calculated after inhibition of de novo mitochondrial and nuclear transcription by the addition of actinomycin D to the cell culture medium. The estimated $t_{1/2}$ of COX III mRNA in control cells were 3 h where as the estimated $t_{1/2}$s of 12S rRNA and of β-actin mRNA were greater than 30 h. Thus, the half-lives of mtDNA-encoded mRNAs are short. These estimated half-lives are similar to the results reported in other cell culture systems (Gelfand and Attardi, 1981; Chrzanowska-Lightowlers et al., 1994). In ouabain- and monensin-treated cells the estimated $t_{1/2}$ decreased to 1.6 and 1 h, respectively. Thus, there is a threefold decrease in the stability of COX III mRNA in drug-treated cells. This posttranscriptional mechanism operating in both ouabain- and monensin-treated cells is likely to be responsible for the accelerated degradation of mtDNA-encoded mRNA. The precise mechanism that mediates a decrease in the stability of

COX III mRNA remains unknown. RNA binding proteins that function to protect mRNA from degradation are well documented (Jackson, 1993). The presence of an abundant mitochondrial RNA-binding protein has been known (Dekkeret al., 1991). Both ouabain and monensin induce an increase in intracellular sodium ion concentration $[Na^+]_i$ in a number of cell cultures including PC12 cells (Pressman and Fahim, 1982; Boonstra et al., 1983; Shier and DuBourdieu, 1992; Blaustein and Lederer, 1999). Increased $[Na^+]_i$ causes subsequent changes in intracellular Ca^{2+}, osmolarity, pH, or a combination of the above. The mechanism by which the increased $[Na^+]_i$ and associated cellular changes influence the stability of mtDNA-encoded mRNA remains to be determined.

In summary, we suggest that there are likely to be at least two mechanisms involved in the regulation of mitochondrial gene expression. A physiological mechanism of regulation that operates at the transcriptional level that would allow mtDNA to synthesize the optimal level of mRNA in response to energetic demands (Enriquez et al., 1996b). The results presented here may represent a second mechanism of regulation that operates at the level of stability of mRNA that is independent of the energetic status of the cell and that may operate under pathologic conditions. This mechanism is likely to be pathological because this overrides the normal regulation by energy demand, causes accelerated degradation of transcripts, and is counterproductive to the actual energy demand of the cell. The results provide a potential approach to determine the molecular components of this mechanism. It is also of interest to determine whether this mechanism contributes to neuronal death in acute and chronic neurodegenerative diseases. To the best of our knowledge, the present study is the first to demonstrate that mitochondrial gene expression is also regulated at the level of mRNA stability under conditions of chronic exposure to elevated intracellular sodium.

Acknowledgements

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Topic 2:   Excitatory amino acids: exicotoxicity and cell death

Abstract Title: SEIZURE SEVERITY AND HIPPOCAMPAL DAMAGE FOLLOWING KAINATE ARE DISSOCIATED AFTER ESTROGEN BUT NOT PROGESTERONE TREATMENT IN RATS

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Key words: EXCITOTOXICITY, EPILEPSY, OVARIAN STEROIDS, LIMBIC SYSTEM

Abstract: Status epilepticus, left unchecked, can produce profound neural damage to the hippocampal formation (HIPP). While increased estrogen (E) lowers and increased progesterone (P) raises behavioral seizure thresholds, it is unclear whether E or P affects neural damage independent of its effect on seizure thresholds. To address this question, ovariectomized female rats were implanted with blank capsules or varying doses of E or P. Seven days later, the rats were administered kainic acid (8.5 mg/kg, ip) and monitored for seizure activity over the next 6 hr. After a 5 day survival, the rats were killed, perfused and their brains processed for localization of neuron nuclear antigen (NeuN). The HIPP was examined throughout its length and scored for severity (0-5) and spread (1-4) of neuron damage. P reduced seizure severity at plasma levels <40 ng/ml but failed to do so when levels were >60 ng/ml. E, at low levels reduced seizures (p<.001) but at high levels, failed to significantly alter KA seizure severity. A strong linear relationship between seizure severity and both the degree (p<.0001; r=0.752) and spread (p<.0001; r=0.818) of neuron loss was noted for P-treated animals. After E, the relationship was lost between seizure severity and the degree (p=.362; r=0.221) or spread (p=.430;r=0.191) of HIPP damage. Moreover, in the E-treated rats, the presence of seizures at any level produced only limited HIPP damage. These data are consistent with the hypothesis that P produces its effects principally by reducing seizures, whereas E is capable of protecting the HIPP from seizure damage.
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Pre-lethal inhibition of mitochondrial gene expression by elevating neural cell sodium with monensin, ouabain, veratradine or excitotoxic levels of glutamate and kainate

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Altered mitochondrial expression likely contributes to the pathophysiology of neurodegenerative diseases. To examine regulation of expression, we developed cell stress paradigms that elevate intracellular sodium ($[Na_i]$) and alter cellular energy demand. Northern blot analysis was used to measure the levels of mitochondrial DNA (mtDNA)-encoded NADH dehydrogenase subunits 1-6 and cytochrome oxidase subunits I-III mRNAs. In differentiated PC12 cells, addition of ouabain (1mM), a Na+/K+-ATPase inhibitor, caused a 50% decrease in mtDNA-encoded mRNAs within 6h. Addition of monensin (0.1 µM), a sodium ionophore, almost totally abolished mtDNA-encoded mRNAs within 6h. The decrease in mtDNA-encoded mRNAs was observed in the absence of significant changes in mtDNA. Transient exposure of primary cultures of rat cerebellar granule neurons to glutamate (100 µM for 30 min) or kainate (100 µM for 30 min) or veratridine (40 µM for 30 min) resulted in a ~50% reduction in mtDNA-encoded mRNA within 6h in the absence of acute cell death. The decreases in mtDNA-encoded mRNAs were observed in the presence of inhibitors of transcription suggesting a mechanism of posttranscriptional regulation of mitochondrial gene expression. Measurement of ATP/ADP ratio in both cell culture systems showed that the decrease in mtDNA-encoded mRNAs was unrelated to the energetic status of the cell. Sodium-dependent reduction in mitochondrial gene expression may increase neuronal vulnerability to stress and contribute to the pathophysiology of neurodegenerative disorders. Supported by grants from AHA 0051001U and US Army DAMD17-99-1-9483.

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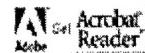
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Title: **Inhibition of mitochondrial gene expression by elevating neural cell sodium with monensin, ouabain, veratradine or excitotoxic levels of glutamate and kainate.**

Body: Evidence suggests that altered regulation of mitochondrial gene expression contribute to impaired energy metabolism in acute and chronic neurodegenerative disorders. To examine regulation of expression, we developed cell stress paradigms that elevate intracellular sodium ($[Nai]$) and alter cellular energy demand. Northern blot analysis was used to measure the levels of mitochondrial DNA (mtDNA)-encoded NADH dehydrogenase subunits 1-6 and cytochrome oxidase subunits I-III mRNAs. In differentiated PC12 cells, addition of ouabain (1mM), a Na+/K+-ATPase inhibitor, caused a 50% decrease in mtDNA-encoded mRNAs within 6h. Addition of monensin (100 nM), a sodium ionophore, almost totally abolished mtDNA-encoded mRNAs within 6h. The decrease in mtDNA-encoded mRNAs was observed in the absence of significant changes in mtDNA. Transient exposure of primary cultures of rat cerebellar granule neurons to glutamate (100 uM for 30 min) or kainate (100 uM for 30 min) or veratridine (40 uM for 30 min) resulted in a ~50% reduction in mtDNA-encoded mRNA within 6h in the absence of acute cell death. The decreases in mtDNA-encoded mRNAs were observed in the presence of inhibitors of transcription suggesting a mechanism of posttranscriptional regulation of mitochondrial gene expression. Measurement of ATP/ADP ratio in both cell culture systems showed that the decrease in mtDNA-encoded mRNAs was unrelated to the energetic status of the cell. Sodium-dependent reduction in mitochondrial gene expression may increase neuronal vulnerability to stress and contribute to the pathophysiology of neurodegenerative disorders. Supported by grants from AHA 0051001U and US Army DAMD17-99-1-9483.

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